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Phosphatidylcholine Biosynthesis in the Neuroblastoma-Glioma Hybrid Cell Line NG108-15: Stimulation by Phorbol Esters

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Abstract: We have examined the effects of phorbol esters on phosphatidylcholine (PtdCho) metabolism in the neuroblastoma-glioma hybrid cell line NG108-15. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), 100 n*M*, stimulated twofold the incorporation of [^{3}H]choline into PtdCho during 2 h of incubation at 37°C. This effect of TPA was concentration dependent, exhibiting an EC_{50} of 24.5 ± 4.4 n*M*. The effect of TPA was also time dependent and became apparent only after a lag period of 15–30 min. TPA also decreased the incorporation of [^{3}H]choline into water-soluble cellular constituents in a manner whose concentration and time-dependence paralleled the changes observed in PtdCho content. HPLC analysis of this pool revealed that the levels of its major (85–95%) constituent, [^{3}H]phosphocholine, were decreased by 29 ± 5%, whereas those of [^{3}H]glycerophosphocholine (0.5–2% of the pool) were increased by 84 ± 4%. PtdCho labeling was also stimulated when cells were pulse labeled with [^{3}H]choline and chased in the presence of TPA. The incorporation of [^{3}H]inositol, [^{14}C]ethanolamine, or [^{14}C]serine into phospholipids was not affected by TPA. The non-tumor-promoting compounds phorbol and 4α-phorbol-12,13-didecanoate (at 100 n*M*) were completely ineffective in modulating choline incorporation, whereas the biologically active analogs 4β-phorbol-12,13-didecanoate and 4β-phorbol-12,13-dibutyrate were as effective as TPA. We conclude that tumor-promoting phorbol esters can modulate PtdCho metabolism in neural-derived cells. The mechanisms mediating this effect and the possible involvement of PtdCho metabolism in normal signal transduction events and in the biological actions of tumor promoters are discussed. **Key Words:** Tumor promoters—Diacylglycerol—Protein kinase C—Choline—Membrane biogenesis—Mitogenesis. **Liscovitch M. et al.** Phosphatidylcholine biosynthesis in the neuroblastoma-glioma hybrid cell line NG108-15: Stimulation by phorbol esters. *J. Neurochem.* **47,** 1936–1941 (1986).

Cell membrane phospholipids participate in cellular responses to a variety of external stimuli. Beside promoting the deacylation of membrane phospholipids and the formation of arachidonic acid metabolites, many hormones and neurotransmitters also cause receptor-mediated changes in the activity of a membrane polyphosphoinositide phosphodiesterase, forming products—inositol phosphates and diacylglycerol—that may act as second messengers within the cell (reviewed by Berridge and Irvine, 1984; Nishizuka, 1984). Inositol trisphosphate may trigger Ca^{2+} release from the endoplasmic reticulum (Berridge and Irvine, 1984), whereas diacylglycerol activates protein kinase C, a Ca^{2+}- and phospholipid-dependent enzyme (Nishizuka, 1984). Phorbol esters, a family of diterpenoid tumor promoters isolated from croton oil (Hecker, 1971), can potently and directly activate protein kinase C by mimicking the action of diacylglycerol (Castagna et al., 1982), an observation implicating activation of protein kinase C in molecular mechanisms mediating tumor promotion.

Phorbol esters exert a wide variety of effects in many cell types (Weinstein et al., 1979; Weinstein, 1981). Among the earliest metabolic responses to these tumor promoters is a rapid and rather selective stimulation of the metabolism of phosphatidylcholine (PtdCho), the major membrane lipid component in eukaryotic cells. Initial observations that [^{3}H]choline incorporation into phospholipids could be stimulated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the most active principal component isolated from croton oil (Hecker, 1971), were made using HeLa cells (Süss

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Abbreviations used: Me_2SO, dimethyl sulfoxide; PBt_2, 4β-phorbol-12,13-dibutyrate; 4α-PDD, 4α-phorbol-12,13-didecanoate; 4β-PDD, 4β-phorbol-12,13-didecanoate; PtdCho, phosphatidylcholine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

et al., 1971). These observations were later confirmed in mouse skin in vivo (Kreibich et al., 1971) and in other cultured cells (e.g., Kinzel et al., 1979). It is of interest that stimulation of PtdCho synthesis was also shown in the human promyelocytic leukemia cell line HL-60 (Cassileth et al., 1981), in which TPA is not mitogenic but, rather, causes cellular differentiation. These results suggest that the early changes in PtdCho metabolism may reflect a primary event in the cellular response to tumor promoters, as well as to other extracellular messengers that use the polyphosphoinositide signal transduction pathway. Yet, the mechanism whereby phorbol esters stimulate PtdCho metabolism, the cellular location where newly synthesized PtdCho is inserted, the resultant changes in membrane composition that may occur, and the functional correlates of these changes all remain obscure.

We have examined the effects of phorbol esters on PtdCho metabolism in the neuroblastoma-glioma hybrid cell line NG108-15. This line offers the advantages of being (a) well characterized and widely studied (Hamprecht, 1977), (b) able to grow in a completely defined, serum-free medium (N2; Bottenstein and Sato, 1979), and (c) able to undergo a striking morphological and biochemical differentiation on specific stimuli (Nirenberg et al., 1983). Here, we demonstrate that phorbol esters stimulate PtdCho biosynthesis in this neuron-derived cell line. Parts of this study have previously been published in abstract form (Liscovitch et al., 1985*a*).

MATERIALS AND METHODS

Materials

[*methyl*-^{3}H]Choline chloride (80 Ci/mmol), *myo*-[^{3}H]-inositol (16.5 Ci/mmol), [*methyl*-^{14}C]cytidine diphosphocholine (42 Ci/mol), [*acetyl*-^{14}C]acetylcholine iodide (2.3 Ci/mol), and L-[^{14}C]serine (169 Ci/mol) were purchased from New England Nuclear (Boston, MA, U.S.A.). [^{14}C]Ethanolamine HCl (95 Ci/mol) was obtained from ICN Radiochemicals (Irvine, CA, U.S.A.). [^{3}H]Glycerophosphocholine, [^{3}H]betaine, and [^{3}H]phosphocholine standards were prepared in our laboratory as previously described (Liscovitch et al., 1985*b*). Phorbol and phorbol esters were purchased from Pharmacia P-L Biochemicals (Piscataway, NJ, U.S.A.) and Sigma (St. Louis, MO, U.S.A.). Stock solutions of phorbol and phorbol esters [10 m*M* in dimethyl sulfoxide (Me_2SO)] were kept frozen at −20°C.

Cell culture

NG108-15, a neuroblastoma-glioma hybrid cell line, was kindly provided by Dr. M. Nirenberg (National Institutes of Health, Bethesda, MD, U.S.A.). Cells (passages 17–30) were cultured in Falcon 200-ml tissue culture flasks under a humidified atmosphere of 90% air/10% CO_2 in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, U.S.A.) containing 100 μM hypoxanthine, 1 μM aminopterine, 16 μM thymidine (all from Sigma), and 5% fetal calf serum (GIBCO). For experiments, cells were subcultured in Falcon 6- or 24-well plates at a concentration of 2.5×10^4 cells/ml. Routinely, media were changed 3–4 days after plating and daily thereafter, and experiments were performed on the subconfluent cultures on days 4–6.

Metabolic labeling and cell extraction

Cells were incubated for 2 h at 37°C with [^{3}H]choline (1 μCi/ml; 30 μM) in the presence of 0.01% Me_2SO alone (vehicle control) or with phorbol ester (100 n*M*), unless indicated otherwise. Following incubation, the plates were placed on ice, radioactive medium was aspirated and each well was rinsed twice with 2 volumes of cold Dulbecco's modified Eagle's medium. Ice-cold methanol (0.7 ml) was then added to each well, and the cells were scraped from the plates and sonicated. Chloroform (2 volumes) and water (1 volume) were added, and the mixture was vortex mixed for 10 s. The upper phase, containing the water-soluble choline metabolites, and the lower phase, containing choline glycerophospholipids, were separated. Radioactivity was determined in aliquots taken from these phases by liquid scintillation spectrometry in a Beckman LS-7500 spectrometer. The remainder of each sample was dried by centrifugation under vacuum and kept at 4°C for further analysis. Protein content was determined in aliquots of the total cell sonicate according to the procedure of Lowry et al. (1951).

Analysis of labeled cellular choline metabolites

Labeled water-soluble choline metabolites present in cell extracts were analyzed by HPLC on a normal-phase silica column, as described in detail elsewhere (Liscovitch et al., 1985*b*), with one modification: To obtain a completely volatile mobile phase, ammonium acetate was substituted for sodium acetate. The retention time of most metabolites is not significantly affected by this modification (cf. Liscovitch et al., 1985*b*); the phosphocholine peak, however, is retarded and elutes at 44–45 min. Metabolically labeled peaks were routinely identified by comparison of their retention times with those of standards chromatographed under identical conditions. TLC of the phospholipid fraction was performed on LK6D plates (Whatman, Clifton, NJ, U.S.A.) using a mobile phase containing chloroform/methanol/isopropanol/0.25% KCl/triethylamine (30:9:25:6:18 by volume) (Touchstone et al., 1980).

Statistics

Experiments were done in duplicate, triplicate, or quadruplicate as indicated and were repeated at least three times. Data obtained in a representative experiment are presented as mean ± SD values of replicates or, when results were obtained in several independent experiments, as mean ± SEM values. The statistical significance of differences was estimated by paired *t* test. EC_{50} (effective concentration for response at 50% of maximum) values were derived from concentration-response curves by a nonlinear, least squares curve-fitting procedure, using a general form of the logistic function (De Lean et al., 1978).

RESULTS

NG108-15 cells incorporated [^{3}H]choline into both chloroform-soluble glycerophospholipids and water-soluble compounds. The effects of different concentrations of TPA on incorporation of [^{3}H]choline into these fractions are shown in Fig. 1. During 2 h of incubation with [^{3}H]choline, TPA increased incorporation of [^{3}H]choline into phospholipids as much as 268 ± 41% (mean ± SEM, n = 5) of control values and

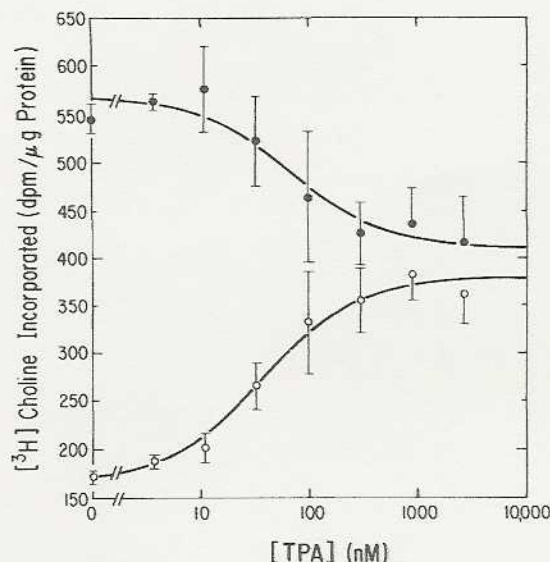

FIG. 1. Variation in the incorporation of [^{3}H]choline into cellular phospholipid (O) and water-soluble (●) fractions as a function of TPA concentration. Cells were incubated for 2 h at 37°C in 0.5 ml of growth medium containing 0.5 μCi of [^{3}H]choline, 0.27% Me_2SO (vehicle), and increasing concentrations of TPA as indicated. The cells were then extracted, and radioactivity was determined as described in Materials and Methods. Data are mean ± SD (bars) values from triplicate wells.

caused a parallel decrease in its incorporation into the water-soluble fraction. TLC of the phospholipid extract revealed that formation of both [^{3}H]PtdCho (which made up >95% of the labeled lipids) and its deacylated derivative [^{3}H]lysoPtdCho exhibited increased labeling (data not shown). The effect of TPA on incorporation of [^{3}H]choline into phospholipids was dose dependent in the range of 3–300 n*M;* higher concentrations (0.9 and 2.7 μ*M*) did not cause any further change. The EC_{50} of TPA was 30 ± 6 n*M* (mean ± SEM, n = 5). The decrease in incorporation of choline into water-soluble compounds was also concentration dependent (Fig. 1) and exhibited a comparable EC_{50} of 42.5 ± 9 n*M* (mean ± SEM, n = 4).

These results strongly suggested that TPA stimulates the utilization of water-soluble PtdCho precursors for PtdCho biosynthesis, without significantly affecting the transport of choline into the cells. In other experiments, no effects of TPA on [^{3}H]choline uptake into NG108-15 cells were observed (Liscovitch et al., unpublished data). However, the increased labeling of PtdCho might have been due to a TPA-induced increase in the specific radioactivity of any of its water-soluble precursors in the absence of any change in the rate of PtdCho synthesis. To test this possibility, the action of TPA on [^{3}H]choline incorporation into PtdCho was examined under chase conditions, in which the initial specific radioactivity of the precursor pool in control and TPA-treated cells is the same. As shown in Fig. 2, the rate of PtdCho labeling during the chase was markedly stimulated in the presence of TPA (after a lag of 15–30 min), a result indicating that TPA indeed affects the rate of PtdCho biosynthesis.

For identification of the enzymatic step in PtdCho biosynthesis that is affected by TPA, the labeled water-soluble choline metabolites extracted from control and TPA-treated cells were separated by HPLC and quantitated (Fig. 3). The major labeled water-soluble species was phosphocholine, which constituted 85–95% of the total, whereas choline represented 2–5%. Betaine and glycerophosphocholine were detectable, but each made up <1–2% of the total, and unidentified counts amounted to 2–7%; CDP-choline and acetylcholine were usually undetectable. TPA treatment significantly reduced the levels of [^{3}H]phosphocholine (by 29 ± 5%, mean ± SEM, n = 4, $p < 0.01$). Concurrently, a significant increase in the levels of [^{3}H]glycerophosphocholine (by 84 ± 4%, mean ± SEM, n = 4, $p < 0.001$) was observed, reflecting, presumably, the increased specific radioactivity of its precursor, PtdCho. Levels of [^{3}H]choline and [^{3}H]betaine were not significantly affected by TPA treatment.

In an examination of the specificity of these responses, the effect of TPA on incorporation of other polar headgroups into phospholipids was examined (Fig. 4). A TPA concentration (100 n*M*) that stimulated [^{3}H]choline incorporation into PtdCho (Fig. 4A)

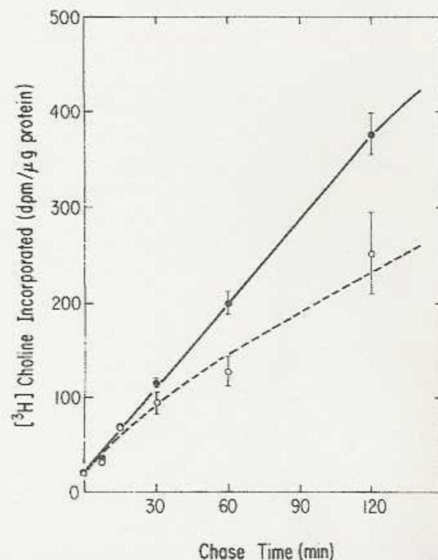

FIG. 2. Effect of TPA on pulse-chase labeling of choline glycerolipids. Cells were pulse-labeled by incubation in 0.5 ml of growth medium containing [^{3}H]choline (2.5 μCi) for 30 min at 37°C. The medium was then replaced with growth medium containing only unlabeled choline (30 μ*M*) in the presence of 0.01% Me_2SO alone (control; O) or with TPA (100 n*M;* ●). The cells were further incubated for the indicated time and then were extracted, and radioactivity was determined as described in Materials and Methods. Data are mean ± SD (bars; half range) values from duplicate wells. The absence of an error bar indicates an SD smaller than the symbol size.

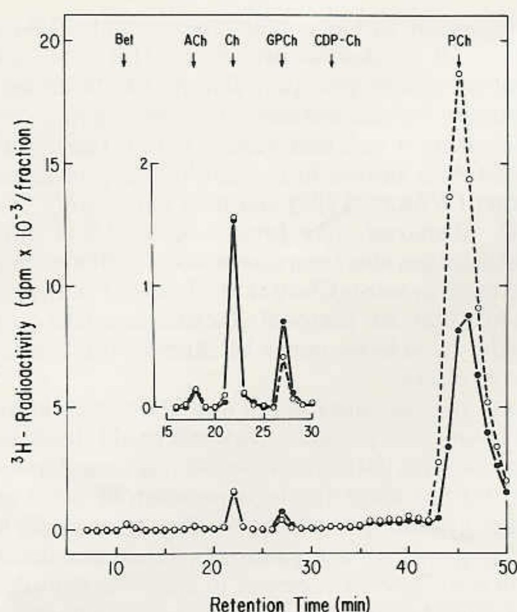

FIG. 3. HPLC of [^{3}H]choline-labeled water-soluble metabolites extracted from control and TPA-treated cells. Cells were incubated for 2 h at 37°C in 0.5 ml of growth medium containing [^{3}H]choline (1 μCi) in the presence of 0.01% Me_2SO (○) alone or with TPA (100 n*M*; ●). The cells were then extracted as described in Materials and Methods. The dried water-soluble extract was dissolved in 50 μl of water, and 20 μl was analyzed by HPLC as detailed in Materials and Methods. Recovery of radioactivity from the column was usually >95%. Two representative radiochromatograms made from samples that differed by <5% in their protein content are presented. Arrows indicate the positions at which betaine (Bet), acetylcholine (ACh), choline (Ch), glycerophosphocholine (GPCh), CDP-choline (CDP-Ch), and phosphocholine (PCh) standards are eluted. Part of the radiochromatogram (15–30 min) is shown in an expanded scale **(inset)**.

failed to affect the incorporation of [^{14}C]ethanolamine (Fig. 4B), [^{3}H]inositol (Fig. 4C), and [^{14}C]serine (Fig. 4D) into phospholipids. Two other potent phorbol esters, 4β-phorbol-12,13-didecanoate (4β-PDD) and 4β-phorbol-12,13-dibutyrate (PBt_2), were as effective as TPA in altering the pattern of [^{3}H]choline incorporation (Table 1), whereas the biologically inactive stereoisomer of 4β-PDD, 4α-phorbol-12,13-didecanoate (4α-PDD), as well as the parent compound, phorbol, were completely ineffective.

DISCUSSION

It has previously been reported that the NG108-15 cell line can incorporate choline (Yavin and Zutra, 1979) and [^{3}H]choline (de Blas et al., 1984) into PtdCho. We have now shown that the latter process is accelerated twofold by tumor-promoting phorbol esters. This effect is probably not due to an effect of these agents on the specific radioactivity of the water-soluble precursor pool, as TPA failed to affect [^{3}H]choline uptake and substantially reduced the radioactivity present as the major water-soluble intermediate, phosphocholine (Fig. 3). Moreover, TPA also was able to stimulate incorporation of [^{3}H]choline into choline phospholipids under pulse-chase conditions (Fig. 2), under which the initial specific radioactivity of the water-soluble precursor pool would have been identical in both control and TPA-treated cells.

Phorbol esters are lipophilic compounds that can intercalate into the phospholipid bilayer (Jacobson et al., 1975). However, their effects on [^{3}H]choline incorporation do not seem to result from such a general membrane perturbation. This conclusion may be drawn from the saturable nature of the effects (Fig. 1), as well as from the fact that only biologically active phorbol esters were active (Table 1). For example, the potent tumor promoter 4β-PDD affected [^{3}H]choline incorporation, whereas its equally lipophilic but biologically inactive stereoisomer, 4α-PDD, was ineffective. In addition, TPA stimulated precursor incorporation into PtdCho but did not affect incorporation of [^{14}C]ethanolamine, [^{14}C]serine, and [^{3}H]inositol into phosphatidylethanolamine, phosphatidylserine, and the phosphoinositides, respectively (Fig. 4), a result indicating that the phospholipid response is not gen-

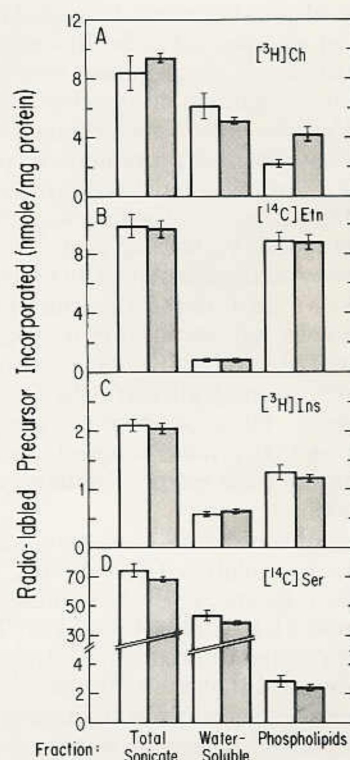

FIG. 4. Effect of TPA on incorporation of [^{3}H]choline **(A)**, [^{14}C]ethanolamine **(B)**, [^{3}H]inositol **(C)**, and [^{14}C]serine **(D)** into cellular lipid and water-soluble fractions. Cells were incubated for 2 h at 37°C in 0.5 ml of growth medium in the presence of 0.01% Me_2SO alone (open columns) or with TPA (100 n*M*; shaded columns) and containing [^{3}H]choline (0.5 μCi), [^{14}C]ethanolamine (0.5 μCi), [^{3}H]inositol (2.5 μCi), or [^{14}C]serine (0.5 μCi). The cells were then extracted, and radioactivity was determined as detailed in Materials and Methods. Data are mean ± SD (bars) values from triplicate wells.

TABLE 1. *Effects of phorbol and various phorbol diesters on [^{3}H]choline incorporation into cellular lipid and water-soluble fractions*

Treatment	[^{3}H]Choline incorporation	
	Phospholipid fraction	Water-soluble fraction
Me_2SO	149 ± 15 (100)	517 ± 35 (100)
TPA	288 ± 39 (193)	370 ± 45 (72)
4α-PDD	154 ± 9 (103)	492 ± 12 (95)
4β-PDD	309 ± 35 (207)	405 ± 23 (78)
PBt_2	276 ± 9 (185)	440 ± 18 (85)
Phorbol	163 ± 4 (109)	495 ± 13 (96)

Cells were incubated for 2 h at 37°C in 0.5 ml of growth medium containing [^{3}H]choline (0.5 μCi) in the presence of 0.01% Me_2SO alone or with the indicated phorbol derivatives (all at 100 n*M*). The cells were then extracted, and radioactivity was determined as described in Materials and Methods. Data are mean ± SD values, in dpm/μg of protein (%), of quadruplicate wells.

eral and raising the interesting possibility that these tumor promoters may cause a change in the lipid composition of the membrane (cf. Rohrschneider and Boutwell, 1973).

The ability of phorbol esters to stimulate PtdCho biosynthesis in a variety of cells, including, as now shown, cells of neural origin, raises two basic questions: By what mechanism do phorbol esters change PtdCho metabolism, and do such changes participate in their actions as tumor promoters or, perhaps, in physiological events associated with transmembrane signaling? Phorbol esters initiate many, but not all (Kreutter et al., 1985; Yamamoto et al., 1985), of their cellular effects by activation of protein kinase C (Nishizuka, 1984). We have found that phorbol and 4α-PDD, two analogs that are not tumor promoting and do not bind to (Kikkawa et al., 1983) or activate (Castagna et al., 1982) protein kinase C, also failed to affect PtdCho synthesis. These observations are consistent with the notion that protein kinase C mediates the stimulation by phorbol esters of choline incorporation into PtdCho.

The major pathway of PtdCho biosynthesis in most mammalian cells involves CDP-choline (Kennedy and Weiss, 1956; Kennedy, 1962). Our data, showing that the amount of [^{3}H]choline present as [^{3}H]PtdCho increases in parallel to a decrease in [^{3}H]phosphocholine content, suggest that the enzymatic step stimulated by phorbol esters is distal to choline transport and phosphorylation. This could be the reaction catalyzed by CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) or, alternatively, the last enzymatic step in the CDP-choline pathway catalyzed by CDP-choline:1,2-diacylglycerol phosphocholine transferase (EC 2.7.8.2). There is ample evidence that the cytidylyltransferase step is rate limiting for PtdCho biosynthesis through the Kennedy pathway in a variety of vertebrate cells (Pelech and Vance, 1984). Recent evidence suggests that TPA can stimulate cytidylyltransferase activity in HeLa cells (Pelech et al., 1984) and in a line of rat skeletal myoblasts (Hill et al., 1984). However, direct phosphorylation of the cytidylyltransferase by phorbol ester-activated protein kinase C is unlikely (Cook and Vance, 1985). The cytidylyltransferase is similar to protein kinase C in its ambiquitous (Wilson, 1978) nature (Vance and Pelech, 1984). Moreover, like protein kinase C, cytidylyltransferase has also been shown to be activated by diacylglycerol in vitro (Choy et al., 1979). It is, therefore, possible that its bimodal distribution and, consequently, its activity could be directly modulated by phorbol esters.

Phorbol esters often act as mitogens; stimulation of membrane phospholipid synthesis could, thus, be an obligatory step in the proliferative response. However, TPA did not affect the incorporation of other polar headgroups into phospholipids (Fig. 4), as might have been expected if the increase in [^{3}H]choline incorporation into PtdCho were due to a general stimulation of membrane biogenesis. Moreover, TPA was also shown to stimulate PtdCho synthesis in the human promyelocytic leukemia cell line HL-60 (Cassileth et al., 1981), in which it induces cellular differentiation rather than stimulating cell division. In addition, some polypeptide mitogens cause an early stimulation of PtdCho biosynthesis, whereas others do not (Warden and Friedkin, 1984). Thus, it seems likely that the response described in this article relates to an early event in the mechanism of phorbol ester action and not to the ultimate cellular response to these agents, which may differ among cell types.

TPA was reported to stimulate the release of radioactivity from cells prelabeled with [^{3}H]choline into the medium (Mufson et al., 1981; Guy and Murray, 1982). Recently, we have shown that TPA stimulates the release of [^{3}H]choline from prelabeled NG108-15 cells (Liscovitch et al., manuscript in preparation), an effect that may reflect breakdown of PtdCho and may be causally related to the stimulation of PtdCho synthesis reported here. It remains to be seen whether the early stimulation of PtdCho turnover elicited by phorbol esters could be mimicked in a receptor-mediated fashion by physiological effectors and what role such stimulation plays in their regulation of cellular function.

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